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13. ABSTRACT *(Maximum 200 words)*

Camptothecin analogues have been developed that show enhanced pre-clinical activity against breast cancer cells. These analogues have been synthesized with two fundamental modifications that aid in their effectiveness against breast cancer: (i) they have a glycinate ester of the 20(S)-OH group, which aids in both solubility and stabilization of the drug's lactone ring; and (ii) they possess a 9-chloro substitution, which allows them to covalently link to DNA. We have studied the activity of these compounds against breast cancer cells in a series of *in vitro* tests: growth inhibitory activity, clonogenic survival assays, and inhibition of topoisomerase I catalytic activity. The new analogues, in both growth inhibition and clonogenic assays, are substantially more effective against breast cancer cells than those topoisomerase inhibitors currently being used clinically. Further, the new analogues are potent topoisomerase I inhibitors. Our results indicate that these new camptothecin analogues possess promising activity as potential clinically useful agents against breast cancer.

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TABLE OF CONTENTS

A. INTRODUCTION

A.1 Cancers, Camptothecin and Topoisomerase I

Cancer is the second leading cause of death (just behind cardiovascular diseases) in the United States. More than one million people are diagnosed with cancer and about half of them die each year in the United States. Surgery, radiation and chemotherapy are the three major modalities for the treatment of cancer. Surgery and radiation are very effective in the removal of tumor locally. Since cancer is a disseminated disease, chemotherapy is generally needed for the treatment of metastatic diseases. Although there have been many advances in cancer treatments and many success stories, the most common cancers — breast, colon, lung and prostate — remain rather refractory to treatment.

In 1966, Dr. Monroe Wall and colleagues discovered that camptothecin (CPT) was the active principal of the extract from the stem of the Chinese tree *Camptotheca acuminata* (1). CPT had significant antitumor activity against L1210 murine leukemia (1). Early clinical trials with CPT in the late 1960s showed that this plant alkaloid had activity against a variety of solid tumors (2-4). However, further development was discontinued because of unpredictable and severe myelosuppression, gastrointestinal toxicity and hemorrhagic cystitis.

Further development of topoisomerase I (topo I) inhibitors for cancer therapy was stimulated by the characterization of CPT as a specific topo I inhibitor (5, 6) Eukaryotic topo I engages DNA in a cycle of breakage and religation steps. These topological adjustments are important in local DNA unwinding during DNA synthesis, repair, and transcription. Topo I relaxes DNA supercoiling by making transient single-strand breaks (7, 8). These breaks are coupled with the transient formation of a covalent DNA-enzyme intermediate termed the cleavable complex (5). CPT specifically and reversibly stabilizes cleavable complexes by inhibiting their religation. The mechanism of CPT cytotoxicity is thought to be the consequence of a collision between moving replication forks and CPT-stabilized cleavable DNA-topo I complexes (9-11).

A.2 Irinotecan and Topotecan

There are several topo I inhibitors at various stages of clinical development. Of these, irinotecan (CPT-11) and topotecan have been approved by the Food and Drug Administration. CPT-11 is a pro-drug and must be converted to SN38 by carboxylesterase to exert its antitumor activity (12, 13). Neutropenia and diarrhea are the dose-limiting toxicities of CPT-11 (depending on the schedule) (14, 15). Thrombocytopenia is the dose-limiting toxicity for topotecan (16). CPT-11 has shown antitumor activity in phase II trials for patients with carcinoma of the colon, lung, cervix, and ovary, as well as for patients with non-Hodgkin's lymphoma. Topotecan has demonstrated clinical antitumor activity against advanced small cell lung, ovarian, and non-small cell lung cancers (14, 15). Irinotecan has recently been approved for the second-line treatment of metastatic colorectal cancer in the U.S. and Europe and for other additional indications in Japan. Topotecan was approved for the treatment of patients with ovarian cancer that is refractory to the platinum analogs.

A.3 Design Novel CPT Analogs With Better Therapeutic Advantages Over Irinotecan or Topotecan

Based on several new findings from our collaborative studies with Dr. Wall of the Research Triangle Institute (RTI), we have developed several approaches for the design of DNA topo I-targeted agents and have identified a number of new CPT analogs that we feel have promise as agents with activity against various forms of cancer. There are three potential ways that one might improve the antitumor activity of DNA topo I-targeted agents. We proposed that analogs that have improved lactone stability, could form an irreversible DNA-topo I cleavable complex, and are not cross-resistant to topotecan and irinotecan will be useful in the treatment of human cancers.

It has been well documented that the intact lactone ring of CPT is required for antitumor activity (17-21). We have extensive preclinical and clinical experience working with both irinotecan and topotecan at the Institute for Drug Development. We have documented that both topotecan and irinotecan are inactivated in patients by hydrolysis of the lactone ring (16). Continuous exposure to

either irinotecan or topotecan increases antitumor activity against human tumor colony-forming units (15). Further support of this hypothesis comes from the preliminary hint of activity of topotecan in patients with advanced breast cancer using a 21-day continuous administration schedule (22). All of these results suggest that a CPT analog with greater lactone stability would be an even better antitumor agent.

The second method that may improve the antitumor activity of CPT analogs comes from our most recent data and is based on generating an irreversible DNA-topo I cleavable complex. Normally, the trapping of DNA-topo I cleavable complexes by CPT analogs is rapid and reversible. Dr. Wall has designed the compound 7-chloromethyl-10,11-MD-20(S)-OH CPT (RT006) which could form an irreversible DNA-topo I cleavable complex (23). We have found that at equal concentrations, Dr. Wall's compound kills more tumor cells than are killed by CPT, which produces a reversible cleavable complex. Therefore, this is an exciting strategy that we will pursue further in this proposal as we seek to enhance the activity of CPT analogs against human cancers.

A.4 Hypothesis/Purpose

Our hypothesis is that CPT analogs will have better clinical antitumor activity than irinotecan or topotecan if those CPT analogs (i) have a more stable lactone ring, (ii) are capable of forming an irreversible cleavable DNA-topo I complex, and/or (iii) are not cross-resistant to irinotecan or topotecan .

B. BODY

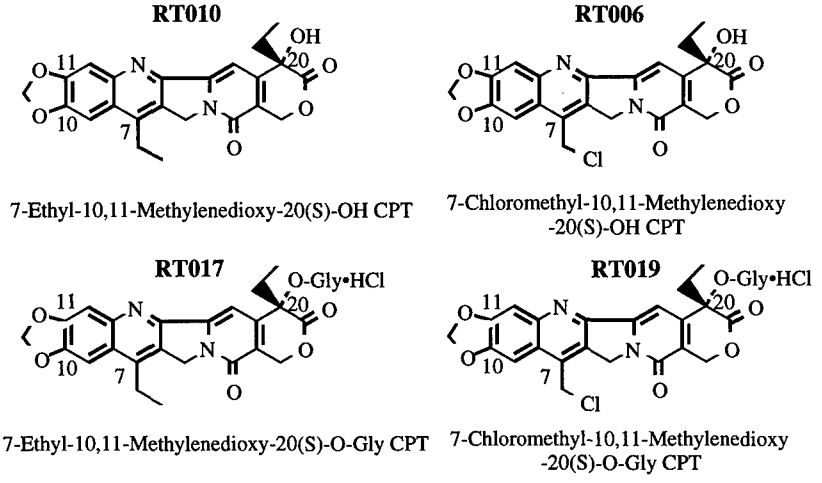
B.1 Compounds of Interest

We have specifically designed and synthesized (with Dr. Wall and his colleagues at the Research Triangle Institute) compound RT019, 7-chloromethyl-10,11-methylenedioxy-20(S)-O-glycinate CPT•HCl, that could fulfill the requirement of being an analog of choice. RT019 contains a 7-chloromethyl group that could trap DNA-topo I cleavable complexes irreversibly and a 20(S)-O-glycinate group that could protect the lactone ring from undergoing pH-dependent hydrolysis. To test our hypothesis, we also prepared and evaluated the following model compounds for comparison:

- RT006: 7-chloromethyl-10,11-methylenedioxy-20(S)-OH CPT
- RT010: 7-ethyl-10,11-methylenedioxy-20(S)-OH CPT
- RT017: 7-ethyl-10,11-methylenedioxy-20(S)-O-glycinate CPT•HCl

RT006 is the parent compound of RT019. RT010 and RT017 contain a 7-ethyl group which theoretically will not form an irreversible DNA-topo I cleavable complex. The chemical structures of these compounds are shown in Fig. 1.

Fig.1 Chemical Structure of RT006, RT010, RT017, and RT019



B.2 Cell Culture

Murine **B16** melanoma cell line was grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 25 µg/ml gentamycin, 0.75% sodium bicarbonate, 10 mM HEPES buffer (pH 7.4), and 0.06 mg/ml AntiPPLO. Murine **P388** leukemic cell line and human **HT-29** colon adenocarcinoma line were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. **P388/CPT** (camptothecin-resistant murine leukemic P388 cell line) was maintained in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, 10 µM β-mercaptoethanol, 10 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamycin. **MCF-7M** human breast adenocarcinoma was maintained in IMEM medium supplemented with 5% non-heat-inactivated fetal bovine serum and 1 nM insulin. **DU145** human prostate carcinoma was maintained in EMEM medium supplemented with 10% non-heat-inactivated fetal bovine serum and 2 mM L-glutamine. **HS578T** human ductal carcinoma was maintained in IMEM medium supplemented in 10% heat-inactivated fetal bovine serum and 10^{-8} M Insulin. **PC3** human prostate adenocarcinoma was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine.

B.3 *In Vitro* Growth Inhibitory Activity

Exponentially growing cells (1-2 x 10^3 cells, unless otherwise specified) in 0.1 ml medium were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1 ml aliquots of medium containing graded concentrations of test analogs were added in duplicate to the cell plates. After incubation at 37 °C in a humidified incubator with 5% CO_2-95% air for 3 days (P388, P388/CPT, B16, HS578T, SK-MES) or 6 days (HT-29, MCF-7M, DU145, PC-3), the plates were centrifuged briefly and 100 µl of the growth medium was removed. Cell cultures were incubated with 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide [MTT, 1 mg/ml in Dulbecco's phosphate buffered saline (PBS)] for 4 hr at 37 °C. The resulting purple formazan precipitate was solubilized with 200 µl of 0.04 N HCl in isopropyl alcohol. Absorbance was monitored in a BioRad Model 3550 Microplate Reader at a test wavelength of 570 nm and a reference wavelength of 630 nm. The absorbance was transferred to a PC 486 computer. The IC_{50} values were determined by a computer program (EZ-ED50) (Perrella Scientific, Inc.) that fits all of the data to the following four-parameter logistic equation:

$$Y = \frac{A_{max} - A_{min}}{1 + (X/IC_{50})^n} + A_{min}$$

where A_{max} is the absorbance of control cells, A_{min} is the absorbance of cells in the presence of highest agent concentration, Y is the observed absorbance, X is the agent concentration, IC_{50} is the concentration of agent that inhibits the cell growth by 50% of control cells (based on the absorbance) and n is the slope of the curve.

B.4 Clonogenic Survival Assay

The MTT assay (described in D.2) measures the ability of the cells to metabolize the MTT; it can determine whether tumor cell growth is inhibited but cannot distinguish whether an agent is cytostatic or cytocidal. We also evaluated the antitumor activity of selected agents by the clonogenic survival assay which measures the ability of tumor cells to form colonies. The tumor cells which survive the drug treatment will continue to proliferate and form a colony with more than 50 cells/colony.

Cultured human breast tumor (MCF-7) cells were exposed to graded concentrations of selected DNA topo I-targeted agents for 1 hr. The cells were harvested by trypsin treatment. The cell pellets were washed twice (to remove residual test agent) with 5 ml medium and were resuspended in growth medium, and the cell concentrations were adjusted for appropriate seeding density. The cells were then replated in fresh medium and incubated at 37 °C for at least six cell doublings, so that more than 50 cells/colony were formed in the control. The cultures were washed with saline and stained with crystal violet (0.5% crystal violet in absolute ethanol). The number of colonies with more than 50 cells/colony were counted. The plating efficiency (PE) was calculated as the percentage of the number

of colonies formed from the total number of cells seeded. Cell survival was calculated as the ratio of plating efficiency of the DNA-topo I targeted agent-treated cells *versus* that of untreated control cells (34, 35).

B.5 Inhibition of Topoisomerase I Catalytic Activity

The topoisomerase I catalytic activity was measured by converting the supercoiled SV40 DNA (Form I) to the relaxed form (Form I_O). All reactions were performed in 20 µl reaction buffer (Tris-HCl, 10 mM, pH 7.5; EDTA, 1 mM; NaCl, 100 mM) with 0.25 µg SV40 DNA, 0.5 unit of human placental topoisomerase I (TopoGen) or calf thymus topoisomerase I (Gibco BRL) and graded concentrations of the analog tested. The reaction mixtures were incubated at 37 °C for 30 min. The topoisomerase I activity was stopped by incubating the reaction mixture with 1 µl of 10% SDS and 1 µl of proteinase K (1.25 mg/ml) for an additional 30 min. One microliter of the loading buffer (1% bromophenol blue and 48% sucrose) was then added. Ten microliters of the reaction mixture were loaded onto a 1% agarose gel prepared in the TAE buffer. The electrophoresis was performed at 82 volts for 3.5 hr in TAE buffer. Another 10 µl of the reaction mixture was loaded onto a 1% agarose gel prepared in TAE buffer containing 2 µg/ml chloroquine; the electrophorsis was performed at 82 volts for 4.5 hr in the TAE buffer containing 2 µg/ml chloroquine. Chloroquine is added to separate nicked and relaxed DNA molecules; without chloroquine, the fully relaxed Form I_O DNA comigrates with the nicked DNA. The gels were then stained with 0.5 µg/ml ethidium bromide solution for 30 min or longer (if chloroquine was present during the electrophoresis step), and destained with 5 changes of deionized water. DNA bands were visualized with a 254 nm uv light (Spectroline Transilluminator Model TL-254A) and documented with Polaroid 665 positive/negative instant pack film. The DNA bands (image) on the negative were densitometrically scanned with a Molecular Dynamic Personal Densitometer. The percent inhibition of topoisomerase I activity is calculated based on the following equation:

$$\%Inhibition = \frac{Fsc_{E+D} - Fsc_E}{Fsc_C - Fsc_E} \times 100$$

where FSC_{E+D} represents the fraction of supercoiled DNA in the presence of enzyme and drug; FSC_E represents the fraction of supercoiled DNA in the presence of enzyme alone; and FSC_C represents the fraction of supercoiled DNA in the untreated SV40 DNA. The IC_{50} value (concentration of drug that inhibits 50% of topoisomerase I activity) was estimated using the same four-parameter logistic equation described in the *in vitro* growth inhibition studies (Section B.3).

B.6 Results

B.6.1 Growth Inhibitory Activity of CPT Analogs

We have found that these two pairs of 20(S)-OH analogs and their corresponding 20(S)-O-glycinate analogs (RT010 *versus* RT017 with a 7-ethyl group, and RT006 *versus* RT019 with a 7-chloromethyl group) have potent and comparable *in vitro* growth inhibitory activity against several cultured murine and human tumor cell lines, including murine leukemia and melanoma as well as human breast, colon, prostate and lung cancer cell lines (Table 1). The growth inhibitory activities of these compounds are at least one to two logs more potent than topotecan or SN38 and at least three logs more potent than irinotecan. The differential cytotoxicity (100-fold) between irinotecan and SN38 is not unexpected, since irinotecan is known as a pro-drug and must be converted to SN38 to exert its cytotoxicity. The data suggest irinotecan is slowly and inefficiently converted (if at all) to SN38 in the presence of cultured tumor cells. On the other hand, the finding that the 20(S)-O-glycinate CPTs and their parent 20(S)-OH CPTs have equivalent growth inhibitory activity (Table 1) suggests that either (i) 20(S)-O-glycinates are active drugs in their own right or (ii) 20(S)-O-glycinates are converted efficiently to 20(S)-OH compounds in the presence of cultured tumor cells.

It is also of interest to note that the 7-ethyl analogs (RT010 and RT017) are generally two to five fold more cytotoxic to cells than the 7-chloromethyl analogs (RT006 and RT019). These results are somewhat surprising and contradictory to what we expected. Based on their mechanism, we expected

that CPT analogs which could trap DNA-topo I cleavable complexes irreversibly would be more cytotoxic. Since tumor cells were incubated continuously with CPT analogs in these studies, the MTT assay may not be able to distinguish the growth inhibitory activity of CPT analogs which trap the DNA-topo I cleavable complex reversibly (RT010, RT017) from those which trap the DNA-topo I cleavable complex irreversibly (RT006, RT019).

Table 1. Growth Inhibitory Activity of RTI Camptothecin Analogs and Standard Agents Against Selected Cultured Murine and Human Tumor Cell Lines

	Growth Inhibitory Activity (IC50; nM)						
Cell Line	RT010	RT017	RT006	RT019	Irinotecan	SN38	Topotecan
B16	0.8 ± 0.1	1.8 ± 0.2	4.6 ± 0.5	8.6 ± 2.3	5148 ± 2828	12 ± 4	63 ± 26
DU145	0.3 ± 0.1	0.4 ± 0.2	1.1 ± 0.6	3.4 ± 1.5	1268 ± 326	3.5 ± 0.5	17 ± 10
HS578T	0.5 ± 0.2	0.7 ± 0.4	1.2 ± 0.1	3.9 ± 1	2165 ± 368	3.3 ± 1	23 ± 1
HT29	0.3 ± 0.1	0.5 ± 0.1	1.3 ± 0.1	2.9 ± 0.4	1552 ± 203	4.6 ± 0.2	24 ± 3
MCF7	53 ± 80	57 ± 90	0.6 ± 0.2	1.2 ± 0.5	88 ± 174	1.3 ± 0.4	12 ± 1
PC3	0.2 ± 0.2	0.3	0.9	nt	15	2.4	nt
SK-MES	0.6 ± 0.2	0.9 ± 0.2	2.3 ± 0.8	5.2 ± 0.7	3253	10 ± 3.7	52 ± 12

B.6.2 Growth Inhibitory Activity Against CPT-Resistant P388 Cell Line

We evaluated the growth inhibitory activity of these CPT analogs against the P388 parental and P388/CPT resistant cell lines. The P388/CPT cell line has been characterized to have an altered form of Topo I and is a generous gift from Dr. J. F. Riou (33). We have demonstrated that CPT is approximately 130-fold less active in the P388/CPT cell line. This cell line is 300-350-fold more resistant to topotecan and SN38 (the active form of irinotecan). However, this cell line is only 25-fold resistant to irinotecan. Since irinotecan is a pro-drug and the 50% growth inhibitory activity of irinotecan against the parental P388 line is already quite high (~4 μM), it is difficult to determine precisely the IC_{50} value of irinotecan against the P388/CPT line. It is of interest to note that all four RTI CPT analogs are considerably more active against the resistant line (~ 7 fold) compared to topotecan and SN38.

Table 2. Growth Inhibitory Activity of RTI Camptothecin Analogs and Standard Agents against Parent and Camptothecin-Resistant P388 Cell Lines

	Growth Inhibitory Activity (IC50, nM)		Resistance Factor*
	P388	P388/CPT	R/S
Camptothecin	17.8 ± 3.8	2356.7 ± 133.2	132
Irinotecan	3967.7 ± 223.8	100310	25
SN38	9.7 ± 1.5	3357 ± 179.6	346
Topotecan	48.8 ± 9.6	14680 ± 8948.4	301
RT010	1.4 ± 0.3	62.4 ± 3.4	45
RT017	1.9 ± 0.3	90 ± 14.1	47
RT006	4.9 ± 1.6	236 ± 121.6	48
RT019	10.2 ± 2.9	953.4 ± 46.3	93

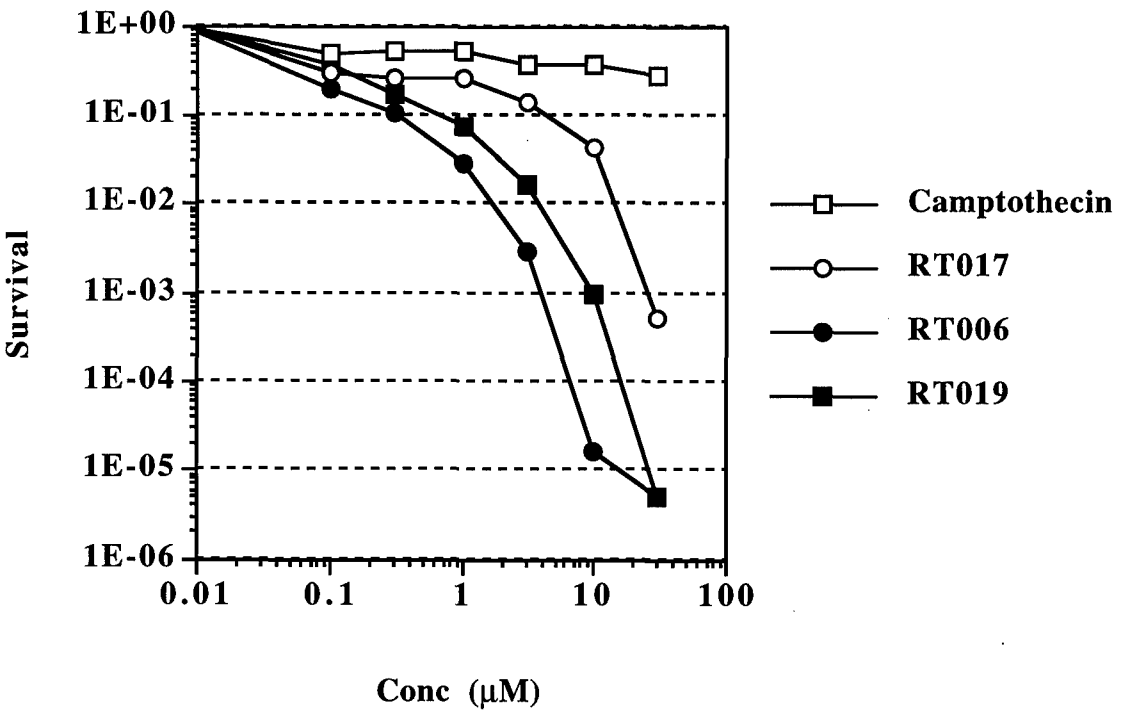
$$\text{* Resistance Factor (R / S)} = \frac{IC_{50} \text{ of P388 / CPT}}{IC_{50} \text{ of P388}}$$

B.6.3. Clonogenic Survival of Tumor Cells Treated With RTI Camptothecin Analogs

In order to determine whether CPT analogs (RT006, RT019) which could trap DNA-topo I cleavable complexes irreversibly are more effective anticancer agents, we performed a clonogenic survival assay. In the clonogenic survival assay, the tumor cells were incubated with CPT analogs for a short period of time (1 hr, in this study). The cells were then washed free of the tested agents, harvested, and replated in a drug-free medium. The cells were allowed to grow in the drug-free medium for an additional 10-14 days. [Note: If drug-induced lesions are reversible or easily repairable in the clonogenic assay, the fraction of cells which survive the initial drug treatment will be greater.]

In the clonogenic survival assay (Fig. 2), we found that 7-chloromethyl-10,11-methylenedioxy-20(S)-OH CPT (RT006) and 7-chloromethyl-10,11-methylenedioxy-20(S)-O-Gly CPT (RT019) killed several logs more MCF-7 (human breast cancer) cells than CPT did. In addition, RT006 and RT019 killed more MCF-7 cells than RT017 at concentrations > 3 μM. RT006 and RT019 have a reactive 7-chloromethyl group, which could form a covalent bond with a nucleophile. Pommier et al. previously demonstrated that 7-chloromethyl-10,11-methylenedioxy-20(S)-OH CPT (RT006) forms a DNA adduct only in the presence of topo I (23). These data support our hypothesis that CPT analogs with the ability to trap DNA-topo I cleavable complexes irreversibly are more cytotoxic than those which trap DNA-topo I cleavable complexes reversibly. These results also demonstrate the importance of using a clonogenic assay to measure tumor cell kill, as the MTT assay only provides a measure of tumor growth inhibition and not quantitative data on tumor cell kill.

Fig. 2 Clonogenic Survival of Human Breast Tumor MCF-7 Cells Treated with Selected CPT Analogs



B.6.4 Inhibition of the Topo I Catalytic Activity by CPT Analogs

Using the cell-free biochemical assay, we determined whether these four CPT analogs inhibit the catalytic activity of a purified topo I enzyme. The catalytic activity of topo I was measured by converting the supercoiled SV40 DNA (Form I) to the relaxed form (Form I_o). All four CPT analogs

demonstrated a concentration-dependent inhibition of topo I catalytic activity . The 20(S)-OH CPTs, RT006 (which forms an irreversible DNA-topo I cleavable complex) inhibits topo I catalytic activity 10-fold less than RT010 (which forms a reversible DNA-topo I cleavable complex). These 20(S)-OH CPT analogs (RT006 and RT010) are at least 10-fold more potent than their corresponding 20(S)-glycinate CPT analogs (RT019 and RT017) in inhibiting topo I catalytic activity. Since these studies are performed under a cell-free system and for only 30 min, 20(S)-O-glycinate hydrolysis is minimized. We conclude that 20(S)-O-glycinates may act like a pro-drug; they are at least 10-fold less potent than their corresponding 20(S)-OH CPTs in inhibiting the topo I catalytic activity. The data are not unexpected, because it has been demonstrated previously by Dr. Wall and other investigators that the 20(S)-OH group of CPT is essential for activity (17, 42) The bulky 20(S)-O-glycinate group might prevent CPT analogs from trapping DNA-topo I cleavable complexes efficiently and, thus, result in a weak inhibition of the catalytic activity of topo I.

C. CONCLUSIONS

We have specifically designed a water soluble camptothecin analog (RT019), 7-chloromethyl-10,11-methylenedioxy-20(S)-O-glycinate CPT•HCl as a potential third generation of camptothecin analogs for the treatment of patient with refractory tumors. This compound processes two essential groups: (i) a 20(S)-O-glycinate group which could protect the E-ring lactone from undergoing pH-dependent hydrolysis and (ii) a 7-chloromethyl group which can trap DNA-topo I cleavable complexes irreversibly.

We compared the biological activity of RT019 to three related compounds (i) RT006, 7-chloromethyl-10,11-methylenedioxy-20(S)-OH CPT, a parent compound of RT019, (ii) RT017, 7-ethyl-10,11-methylenedioxy-20(S)-O-glycinate CPT•HCl, a counterpart of RT019, which could not trap DNA-topo I cleavable complexes irreversibly, and (iii) RT010, 7-ethyl-10,11-methylenedioxy-20(S)-OH CPT, a parent compound of RT017. In addition, we compared the activity of these four CPT analogs with that of irinotecan and topotecan.

In this report, we investigated the growth inhibitory activity of these agents against a variety of murine and human tumor cell lines, and the mechanism of inhibiting topo I activity, by using a purified enzyme and by trapping DNA-topo I cleavable complexes.

We have found that the growth inhibitory activities of the 20(S)-O-glycinate and 20(S)-OH CPTs are remarkably similar (RT010 *vs.* RT017 and RT006 *vs.* RT019). In the growth inhibitory activity studies, the cells were incubated with testing agents continuously for 3-6 days, and the glycinate group may have been hydrolyzed in the cultured medium. Therefore, the glycinate and its parent compound exerted a similar growth inhibitory activity. On the other hand, we were surprised to find that 7-ethyl substituted analogs (RT010 and RT017) demonstrated more potent growth inhibitory activity than their corresponding 7-chloromethyl-substituted analogs (RT006 and RT019). This is somewhat perplexing, since we have speculated that compounds with a 7-chloromethyl group that can form an irreversible DNA-topo I complexes may be more potent antitumor agents. Therefore, it would be very important to understand why compounds which theoretically do not form an irreversible cleavable complex are more potent antitumor agents.

Since tumor cells were incubated continuously with testing agents in the growth inhibitory studies, it is difficult to distinguish the antitumor potential of CPT analogs which trap DNA-topo I cleavable complexes reversibly or irreversibly. We have used a clonogenic survival assay to address whether compounds with the ability to trap DNA-topo I cleavable complexes irreversibly are better antitumor agents. In the clonogenic survival assay, the cells were treated with test agents for 1 hr and were replated in drug-free medium. We found that CPT analogs (RT006 and RT019), which trap DNA-topo I cleavable complexes irreversibly, produced more extensive tumor cell kill than the CPT analog (RT017) which traps DNA-topo I cleavable complexes reversibly. I

In contrast to the results obtained in the growth inhibitory studies, we found that parental CPTs (RT010 and RT006) are one to two logs more potent than their 20(S)-O-glycinate CPTs (RT017 and

RT019) in inhibiting the topo I-catalyzed relaxation of supercoiled DNA . However, some topo I inhibition was observed with the glycinate esters at time points when hydrolysis of the glycinate ester is minimal. This suggests that the glycinate esters may also have intrinsic topo I inhibitory activity, although much less so than the hydrolyzed compounds.

Our experiments thus far suggest that our compounds RT017 and RT019 and their hydrolyzed metabolites are more potent against *in vitro* breast cancers than those topo I inhibitors currently being used clinically. This suggests that these agents may be powerful new antitumor agents for treatment of breast cancer. Our experiments over the upcoming year will focus on two aspects necessary to begin to examine these agents for possible clinical use: (1) mechanistic studies of the cellular toxicities of the compounds, and (2) the activities of the compounds in *in vivo* models of breast cancer.

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